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THE EFFECTS OF POLLUTANT GASES, PARTICLES AND INDUSTRIAL ATMOSPHERES ON LUNG SURFACTANT

Submitted on behalf of

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TABLE OF CONTENTS

		Page
A.	INTRODUCTION	1
	1. Objective	1
	2. Background	1
	3. Work Done by Others	4
	4. Rationale	10
B.	SPECIFIC AIMS	12
C.	METHODS OF PROCEDURE	13
	1. Experiments on Biosynthesis and Turnover of Surfactant	16
	2. Other Determinations	16
	3. Specific Experiments	17
	4. In Vitro Experiments	20
D.	SIGNIFICANCE	21
E.	FACILITIES AVAILABLE	22
F.	REFERENCES	24
G.	BIOGRAPHICAL INFORMATION	29

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A. INTRODUCTION

1. Objectives

Many investigations have been undertaken to elucidate the biosynthetic pathways of surfactant lecithin production and to clarify the in vivo assembly of the surfactant system. This system is currently thought to consist of phospholipid, predominantly dipalmitoyl lecithin, and protein. It has been suggested that these are assembled within Type II alveolar cells and secreted and catabolyzed as a unit. The evidence is chiefly indirect, however.

The surfactant is produced in the lung and is essential to its normal function; the surfactant prevents collapse of air spaces at physiological pressures. Interference with production and subsequent activity of surfactant could lead to higher surface tension. As a result it will require greater pressure to inflate the lung so that pulmonary compliance will be reduced. Interference with surfactant activity leads to abnormality of lung function (respiratory distress syndrome).

It has been speculated that during episodes of high air pollution or exposure to toxic gases there may be a short-lived interference with surfactant activity. Absence of a permanent reduction of surfactant production are primarily based on surface tension measurements, which are based on questionable rationale.

The purpose of the studies described below is to use single pollutant inhalation, synergistic mixtures, industrial atmospheres, and highly polluted atmospheres and dietary manipulation to produce changes in synthetic and secretory rates and to monitor concentration and composition of surfactant to further elucidate basic mechanisms of surfactant activity and metabolism.

2. Background

Under a joint NIH-grant the two principal investigators are currently engaged in a study of the biosynthesis of the surfactant system in vivo and in vitro in order to learn more about relative importance of different pathways, and a study of the effect of tabacco smoke and tobacco smoke particulate fractions on surfactant turnover rates.

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One of us (JAB) and his colleagues at AMC have published a number of papers concerned with phospholipid metabolism in normal and EFA deficient rats and hamsters especially with reference to hepatic and biliary lecithin metabolism (1-6). These studies have demonstrated the existence of several functionally distinct pools of lecithin within the liver (1,2). Furthermore, it was shown that the synthesis, as well as secretion, of biliary lecithin was accelerated by increasing bile salt loads (5). In addition, these studies demonstrated that certain types of lecithin were preferentially synthesized by either the direct Kennedy pathway or the methylation pathway of Greenberg and Bremer (2,4). Also published have been studies on the methodology of phospholipid analysis by column or argentation chromatography (7,8) as well as studies on the neutral fraction and phospholipids in a number of lipid storage diseases (9,10,11).

During the past three years we have been engaged in studies in the metabolism of phospholipids in different tissues of dogs and rats and of the possible effects of exposure to tobacco smoke on these processes. The results of studies in dogs have recently been published (12). The data obtained in these experiments cast doubt on the importance of the methylation pathway to lecithin synthesis in lungs, as they showed no evidence of ^{14}C incorporation into lecithin following injection of methionine-methyl-^{14}C or of ethanolamine-1,2-^{14}C. The data also suggested decreased synthesis of pulmonary surfactant lecithin in dogs exposed to tobacco smoke for short periods of time (2-4 hours). These latter conclusions, however, are open to criticism, as they are based essentially on observations obtained after a single time period only.

More recently we have been engaged in studies of phospholipid metabolism in lungs, heart and liver of rats exposed to tobacco smoke for 2 - 3 days prior to isotope injection and of normal controls. These animals were then followed in groups of 4 for 2 - 48 hours. The data indicate statistically significant increase in the specific activity in surfactant lecithin of ^{3}H-glycerol at 2 hours ($p < .05$) (Fig. 1) in smoked as compared to control animals. Current experiments have

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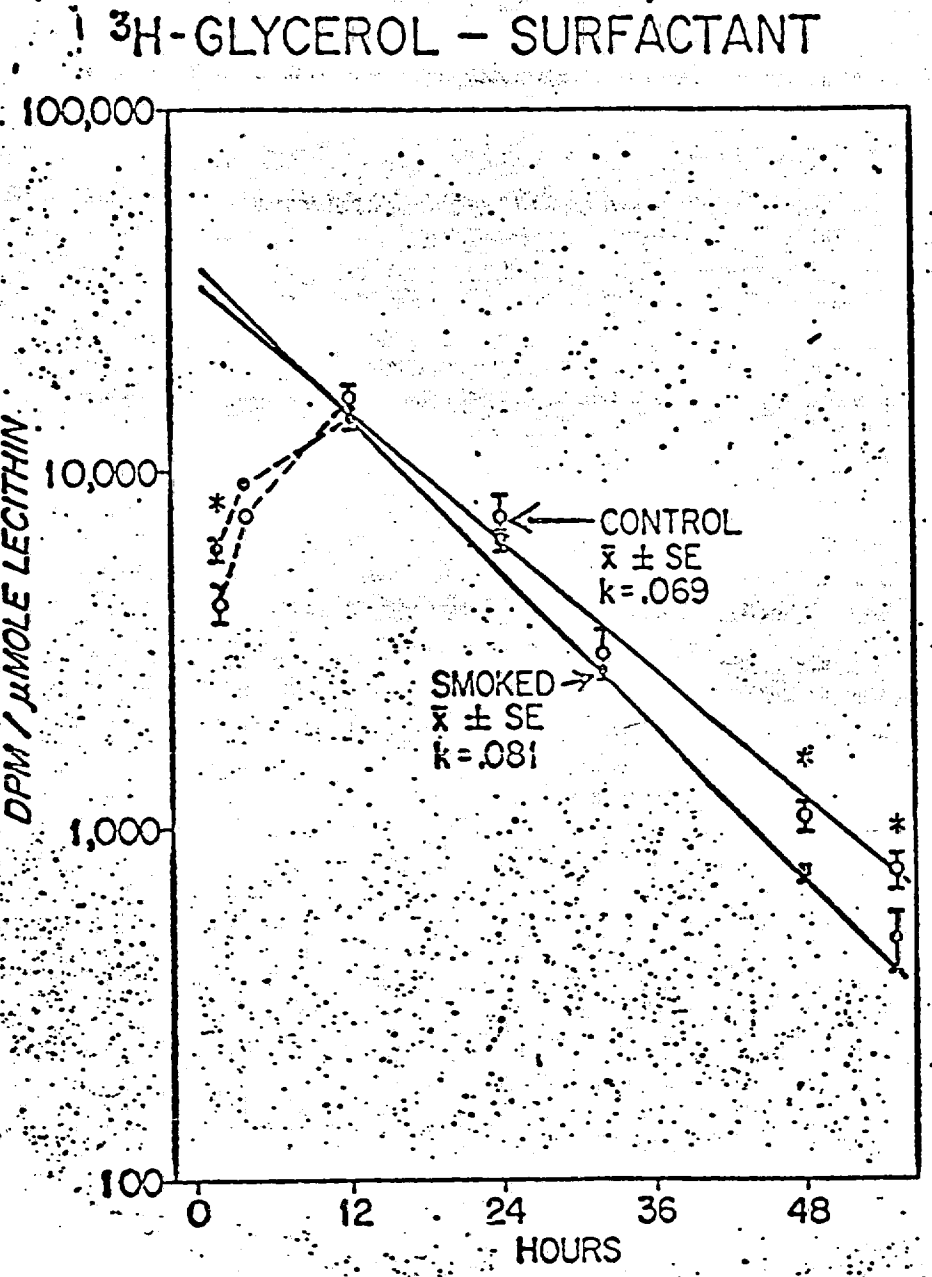

Figure 1

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repeatedly confirmed this finding. This difference is still present though no longer significant at 4 hours, but is reversed at 12 hours. Glycerol-^{3}H specific activity then declines more rapidly in surfactant lecithin from smoked animals than controls so that by 48 hours the difference is again significant ($p < .05$). Data for 32 hours and 60 hours support these findings. The slope for controls = .073 and for smoked animals = .083. Specific activity from choline-1,2-^{14}C is higher in controls than in surfactant lecithin from smoked animals at 2 hours ($p < .05$) and remains higher throughout the 48 hours. (At 24 hours, $p < .05$ and 48 hours, $p < .01$). These results have now been fully confirmed in a second experiment. These data suggest increased turnover of surfactant lecithin in smoke exposed rats with perhaps reutilization of choline. Glycerol labeled in 2 position with ^{3}H is not reused as it is lost in the body water pool. More detailed studies for longer time periods and the use of selectively filtered smoke are currently under way. Data from these studies indicate that surfactant lecithin contains at least two pools, one with a half life of 12-15 hours, and the other with a half life of 50-52 hours in respect to decay of 2-^{3}H-glycerol and 1-^{14}C-palmitate labels.

These long term studies have shown, furthermore, that the turnover of glycerol and palmitate are similar as indicated, and shorter than that of choline-1-2-^{14}C and ^{32}P phosphate. These latter tracers exhibit half lives of 20-25 hours and 70-80 hours for the rapid and slow compartments respectively. Possible reasons for this difference between glycerol and fatty acid on the one hand, and choline and phosphate on the other in lung is being investigated presently in in vitro studies of choline oxidation. It is of interest that no such differences can be demonstrated in liver lecithins.

The other investigator (ERA) and his students have been active in a number of areas in air pollution. Specifically, the effects of additives in fuel oils on the NO_x-formation during combustion has been studied using a small model combustor (13-17). In this connection, extensive investigations into measurements of NO and NO_2 have been made (Saltzman, PDSA, gas chromatography, and mass spectrometry have

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been used). Currently, all our measurements are done via chemiluminescence. Another study has dealt with the kinetics of the ozone/olefin reaction (18) and the use of several measurement techniques for the detection of high and ambient ozone concentration (KI-method, olefin reaction, Mast ozone meter, chemiluminescent ozone monitor). Some studies have been carried out on the measurement of ozone in the presence of sulfur dioxide by these techniques.

Small particle measurements have been carried out in our laboratory by the use of Andersen heads on Hi-Vol samplers, the Lundgren Impactor, the Integrating Nephelometer, and the Condensation Nuclei Counter (CNC). Smoke filtration experiments using mass and CN-counts are currently in progress in connection with the surfactant turnover studies. In the past, a small study on ambient air pollutant measurements in a rural-recreational area (19) have been carried out. In this connection, benzo[a]pyrene was measured by fluorescence.

Another study - just commencing jointly with the Division of Air Resources of New York State Department of Environmental Conservation - is aimed at measurement and identification of ambient air hydrocarbons at a site in Troy, New York and in New York City.

3. Work Done by Others

Recognition of a surface active component of pulmonary edema fluid is very recent and derives from the observations of von Neergaard (20) and Pattle (21,22). Present knowledge concerning the metabolism, nature and disorders of the surfactant system have been extensively reviewed recently (23-25). Surfactant lecithin has been shown to be predominantly (50% or more) dipalmitoyl lecithin (26,27). Watkins (28) showed that this type of lecithin is particularly suitable for a spreading surfactant film due to its packing characteristics.

Morgan et al (26) demonstrated the presence of phosphatidyl dimethyl ethanolamine (PDME) in dog lung and lavage fluid (about 4-5% of total phospholipids) They showed that this compound was rich in palmitic acid and has surface active characteristics similar to those of dipalmitoyl lecithin. They suggested that

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PDME may be an intermediate in the synthesis of surfactant lecithin via the methylation pathway. Morgan (29) went on to demonstrate N-methyl transferase activity in dog lung microsomes. He showed that this enzyme in lung was most active with saturated PE as substrate and catalyzed the synthesis of saturated lecithin from mixed tissue PE. No such specificity was seen in the action of hepatic N-methyl-transferase. He further demonstrated that this enzyme activity was inhibited by high O_2 tensions. This enzyme activity was present in microsomes and in the lamellar bodies. Further evidence for the importance of the methylation pathway for the synthesis of dipalmitoyl lecithin of lung surfactant has come from studies of fetal lung lipid metabolism by Gluck and associates (30,31), Chida and Adams (32) and Morgan (33). These workers showed that just before birth synthesis of dipalmitoyl lecithin increased, together with increased activity of the methylation pathway and appearance PDME in lung extracts.

Other workers have questioned the predominant role of the methylation pathway in the biosynthesis of pulmonary surfactant. Thus, Spitzer et al (27, 34,35) have indicated that <u>in vivo</u>, using isotopically labeled choline, methionine-methyl and $^{32}PO_4$, the CDP-choline biosynthetic pathway is more important in adult female rats. These authors demonstrated very slow turnover of PDME relative to that of lecithin and could not confirm the similarity of fatty acid pattern between PDME and surfactant lecithin. Using similar techniques in dogs, we could not show significant incorporation of methyl group from methionine into pulmonary surfactant lecithin (12). Morgan (25,33) has recently concluded that the CDP-choline pathway may indeed be quantitatively more important under normal conditions. Of interest is a recent study by Pawlowski et al (36) which suggests the presence of a pool of surfactant type lecithin in lung parenchyma, associated with lamellar bodies, which may be the precursor or storage form of surfactant lecithin.

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Several investigators have suggested that the surfactant system contains a lipoprotein and that surfactant lecithin is excreted by the Type II cells in that form. Massaro (37) confirmed the presence of laminar bodies in Type II alveolar cells (granular pneunocytes). He showed that in isolated cells from lung lavage fluid (50% of cells were Type II) labeled glucosamine, galactose and mannose was incorporated into protein in the microsomes and then transferred to the laminar bodies shown to sediment at 15000 g (see also ref. 36). He suggested that this protein was part of the surfactant system. Massaro et al (38) confirmed these results both in vivo and in vitro using rats. They showed that epinephrin increased secretion of protein into the incubation medium in vitro whereas cyanide and cold (0°) inhibited this process. Pruitt et al (39) isolated a fraction from pig lung lavage fluid containing about 20% by weight of protein and rich in dipalmitoyl lecithin. They showed that this protein was immunologically not serum albumin. Similar composition of a fraction obtained from dog lung material has been reported (36). The significance of these data is thus questionable. The data of Spitzer and Norman (35) showing similar turnover of choline, phosphate and leucine in rat lung surfactant seem to support the concept that surfactant is a lipid-protein complex.

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Recent studies by King and Clements (40) have demonstrated the presence of an immunologically distinct protein in lung lavage fluid, which by fluorescent microscopy, can be shown to be on the alveolar surface. This protein furthermore, is isolated from lung lavage material and lung homogenates, with the surface active saturated lecithin. These observations, therefore, present strong evidence to support the contention that surfactant is a lipoprotein.

Recent studies by Naimark (41) using labeled palmitic acid have indicated that alveolar macrophages may play an important role in the catabolism of surfactant lecithin. He showed that after injection of this tracer, radioactivity first appeared in lung DPL, then in surfactant lecithin and then in macrophages.

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However, studies on the turnover of surfactant lecithin are difficult to interpret, because of uncertainty as to which part of the molecule should be followed. Tierney et al (42) calculated a turnover of pulmonary surfactant to be 0.25 μmole lecithin/gm of lung/hr from loss of compliance with rapid breathing. Using ^{14}C palmitate and U-^{14}C-glucose (to label glycerol) they arrived at a turnover rate of 0.3 μmole/gm/hr (42). They observed similar half-lives for pulmonary and surfactant lecithin (about 14 hours). Polyunsaturated lecithins turned over more slowly. Spitzer et al (34,35) obtained a half-life of surfactant lecithin using labeled choline and $^{32}PO_4$ of around 40 hours. Newman and Naimark (43) obtained a half life for surfactant lecithin of about 8-18 hours in normal animals and showed that hypoxia decreased turnover rate. Pilot studies in our laboratories (quoted above) gave a half-life for surfactant lecithin of 11.5 hours in normal animals, (8 hours in those exposed to tobacco smoke) using ^{3}H-glycerol. Half-life of choline-1,2-^{14}C in the surfactant of these animals was 19-20 hours. These findings by different authors would be consistent with the concept of diglyceride exchange but clearly need further detailed evaluation.

Miller and Bondurant (44) showed that tobacco smoke blown over a surface film in a Wilhelmy trough lowered surface activity. Data to show that similar effects may occur <u>in vivo</u> are less convincing though evidence for such an effect in man has been presented (45). Fresolomo et al (46) suggested that surfactant lecithin is bound to protein by non-covalent bonding, thus accounting for its ready dissociation from protein. They considered that lecithin is probably present in liquid crystalline form stabilized by water, protein and counter ions. It is possible that these liquid crystals could thus be precipitated out by seeding with various particles in inspired air. Pulmonary artery occlusion and hypoxia, pulmonary edema and hyperbaric O_2 have been shown to reduce surfactant and lung compliance (for review see 25). Ladman and co-workers (47,48) have demonstrated changes in alveolar macrophages in cigarette smokers, with presence of large inclusions in the cells of the latter. Included in these inclusions were myelin

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figures, which might represent phagocytized surfactant lipoproteins. These findings are consistent with the metabolic studies cited above (41). Thickening of bronchiolar basement membrane has been reported in rats exposed to NO_2 for long periods (49). Similar changes have been reported after prolonged exposure to tobacco smoke (50). Recent work by Rhoades of long term exposure of rats to NO_2 gave evidence of altered surface activity and phospholipid composition of lung washings (51).

Gil (52) has drawn attention to the difficulties inherent to electron microscopic studies of lung tissue and surfactant. However, a number of studies (36,46,47,48) have recently shown that with proper attention to detail good preservation and images may be obtained. This technique has been used to correlate structure and lipid composition (53) and structure and metabolic function (36).

In terms of physico-chemical and photo-chemical interactions between surfactant and atmospheric or industrial pollutants dipalmitoyl lecithin (DPL) and egg lecithin (EL) monomolecular films and their interaction with NO_2/olefin mixtures have been studied (54). Films of the saturated phospholipid (DPL) showed no interaction with any of the test atmospheres, while all NO_2 containing atmospheres effected a change with the unsaturated egg lecithin (EL); the evidence in the latter case was interpreted as a chemical reaction rather than a simple physical penetration phenomenon; contrast this with the findings of Rhoades (51). Since lung surfactant is predominantly DPL, it was speculated that the primary site of attack of pollutants were the materials of the capillary cell membranes rather than the surfactant. It is clear that isolated lung surfactant does contain some unsaturation, however (46,51); this should be susceptible to ozone attack. A recent symposium touched on many of the difficulties and conflicts in studying the interaction of pollutants with various loci within the lung (55). Coffin and his group (55) presented evidence to the effect that exposure to ozone had no significant effect on pulmonary surface activity or fatty acid composition of phospholipids. It was pointed out, however, that the surface tension studies were qualitative in nature

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and that a value of 15 dynes/cm reflected simply an arbitrary evaluation of the "presence" or "absence" of pulmonary surfactant and that quantitative inferences should not be made. In line with the in vitro findings cited above it was shown that at levels of exposure which produced extensive morphological changes in alveolar macrophages, there was no alteration in phospholipid composition or pulmonary surface activity and that alterations in cell structure are not mediated directly through a change in protective function by an agent such as DPL. Gardner et al (55) made similar observations in that the ability of DPL to protect macrophages was lost upon exposure to ozone, but the surface tension-lowering properties were unchanged. Experiments supposedly showing the destruction of surfactant by smog components have been reported (56).

Ramirez et al (55) studied diseased patients and found varying amounts of unsaturated fatty acids in the lipids of washings from such patients. Pattle (57) has speculated on the possible reaction of lung surfactant to nitrogen dioxide; however, cf. ref. 51.

An extensive body of literature exists on ozone and photochemical oxidant toxicology (58,59,60). Specifically, Goldstein (61) has suggested oxidation of fatty acids as a cause of ozone toxicity and pointed out the necessity for studies at ambient pollutant levels to determine whether lipid peroxidation can take place under such conditions in the lungs (62). Roehm (55) reported that oxidation of fatty acid methyl esters was increased by traces of nitrogen dioxide oxidations in films, and retarded both nitrogen dioxide and ozone oxidation in aqueous solutions. It has also been shown that a considerable portion of ozone is removed in the upper airways (63). Effects of surfaces on ozone makes it difficult to calculate the amount actually reaching the alveoli (64,65,66). In man, short-term ozone exposure has shown a significant increase in pulmonary resistance (64).

Changes in chemical composition of lung tissue homogenate, following ozone inhalation, could not be traced to a particular tissue component (67).

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Results on pulmonary deposition of aerosols have been reviewed (68). Experimental and theoretical studies on the breathing of half-micron aerosols by Davies and co-workers have been interpreted as showing the complete absence of mechanical mixing in the alveolated airways (69).

4. Rationale

Current studies in our laboratories have shown that tobacco smoke inhalation accelerates the turnover of pulmonary and surfactant lecithin. Previous studies by others and ourselves show that the phosphoryl choline moiety of surfactant lecithin turns over more slowly than the glycerol and fatty acid portion. This could be explained by the diglyceride exchange postulated by Bjornstad & Bremer (70) or by assuming reutilization of phosphoryl choline. This question can be resolved by long term turnover studies in which the decay of radioactivity is followed in both the lecithin and its water soluble phosphorylated precursors. Long term turnover studies may in fact be the only way in which relationships between surfactant activity and permanent detrimental changes in the lung can be shown. There is evidence that chronic inhalation of irritant materials stimulates a large increase in the number of free alveolar phagocytes and decrease in the amount of extracellular surfactant in the alveoli (71).

The experiments with individual pollutants - some of which will be carried out shortly - will be aimed at the assessment of turnover rates to further substantiate evidence currently being gathered. However, it is unlikely that typical ambient concentrations of single pollutants will show permanent effects (51). It seems more plausible to concentrate on synergistic mixtures (O_3, NO_2; O_3, NO_2, submicron particles; O_3, SO_2, trace metals in particles), highly polluted atmospheres, industrial atmospheres (which may be high in one or more toxic component, such as SO_2, CO or Cl_2, etc.), and special situations (i.e. H_2SO_4 mist which may arise to an increasing extent from automative exhaust catalysts in 1975).

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Although it has been reported that DPL is not altered by ozone (cf. work done by others), this is perhaps somewhat surprising. Ozone does, of course, attack olefinic bonds faster than others, but it also attacks heteroatoms and C-H bonds. Moreover, attack on double bonds or heteroatoms may give rise to radicals which could form unstable hydroperoxides or to singlet oxygen ($^1O_2$). Both types of reactions have been demonstrated in vitro (72,73,74). Pryor (74) has estimated that if all the ozone breathed by a human were converted to radicals, about 10^{-6} mole of radicals would be formed in the human body per day. Undoubtedly, only few of these radicals are involved in damaging processes but the suggestion that the lung mucus reacts with most of the ozone and protects lung tissue by a sacrificial mechanism lacks experimental substantiation and appears to be at variance with some in vivo experiments (cf. work done by others). If morphological changes occur while the material which presumably "guards" the air-lung surface interface remains intact, a sacrifical mechanism is not indicated (49). The turnover experiments and selective microscopic studies now in progress should help to clarify this point.

Certain synergistic combinations could be crucial as far as long term implications are concerned. Witness Selikoff's (75) findings on the synergistic effect of asbestos and cigarette smoke; it is expected that results from control experiments vs. toxic substances may soon be grossly predictable, based on the trend of our data; but it is considerably more difficult to predict which synergistic combinations of two or more substances may lead to higher turnover rates as well as chronic effects.

In addition, we have shown that in animals deficient in essential fatty acids (EFA) uptake of fatty acid by intestinal mucosa and of bile salts by liver is normal; however, removal of triglycerides into intestinal lymph (76) and secretion of bile salts and lecithin into hepatic bile is markedly impaired (6). These results, and similar data by other investigators (72), indicates an excretory defect in EFA deficient animals. It seems likely, therefore that a similar

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excretory defect may be produced in the lungs by EFA deficiency. Such an excretory block may so alter surfactant excretion as to produce more severe lung damage in animals exposed to pollutants. In liver EFA deficiency is associated with increased phospholipid turnover. If similar changes occur in lung, increased incorporation of isotope into intracellular lipids may be found. Simultaneous examination of protein synthesis using labeled amino acids under such circumstances could yield important information concerning the site of assembly of the surfactant system.

Since increased breathing rates can lead to increased turnover rates, this effect will have to be accounted for. Specific experiments to do this are outlined below.

Finally, human studies on turnover rates using stable isotopes are proposed.

Radioactive isotopes have limited usefulness for human studies, such as described above, because of the magnitude of the radiation dose involved in order to obtain reliable count rates. As it is clearly important to confirm animal data in man, we plan to develop the techniques for use of stable isotopes (deuterium and N^{15}) in human studies. We plan to use uniformly D_2 labeled palmitic acid from commercial sources. They are available, but very expensive at present. The biochemical and physiologic studies with stable isotopic tracers will be similar to those used with radioactive isotopes. The techniques, however, will need to be developed. Preliminary work in this approach has been started. (Dr. J. Burnell who recently completed her Ph.D. program at RPI, will join us for the specific purpose of developing these techniques in conjunction with Dr. John Hudson at RPI).

B. SPECIFIC AIMS

1. To obtain clarification of the sites and mode of biosynthesis and secretion and sites and modes of catabolism of surfactant in normal and EFA-deficient rats.

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2. To examine the effect of environmental pollutants, synergistic mixtures, highly polluted atmospheres, and typical industrial atmospheres upon these processes.

3. To study the effect these pollutant mixtures in an isolated heart-lung preparation (78).

4. To study certain substances (or groups of substances) from 2. in combination with cigarette smoke (and fractionated-as to particle size - smoke).

5. To study the effects of these pollutants upon cardiac and hepatic phospholipid metabolism, as controls for the above studies.

6. To study surfactant turnover rates in anesthesized and exercised rats (both controls) and those exposed to toxic substances in order to further delineate the reasons for the increased rates of catabolism.

7. To investigate surfactant turnover in humans using stable isotopes.

8. To develop computer models of the observed metabolic schemes, and thus to try to site of any metabolic effects observed.

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C. METHODS OF PROCEDURE

Various groups of rats will be used for these studies and at least 8 animals in each group will be used to determine the mean value and SD for each time period. The groups of animals will be as follows:

1. Control - animals fed regular chow and not exposed to pollutants.

2. EFA deficient controls - weanling rats fed a fat free diet (Nutritional Biochemicals Company) supplemented with 4% tripalmitin for 12 weeks. Not exposed to pollutants.

3. Animals exposed to pollutants - fed regular chow.

4. EFA deficient animals fed as for 2 but exposed to pollutants for various time periods.

5. An additional control group will receive the fat free diet supplemented with 4% safflower oil, in order to rule out any effects due to difference in dietary carbohydrate content.

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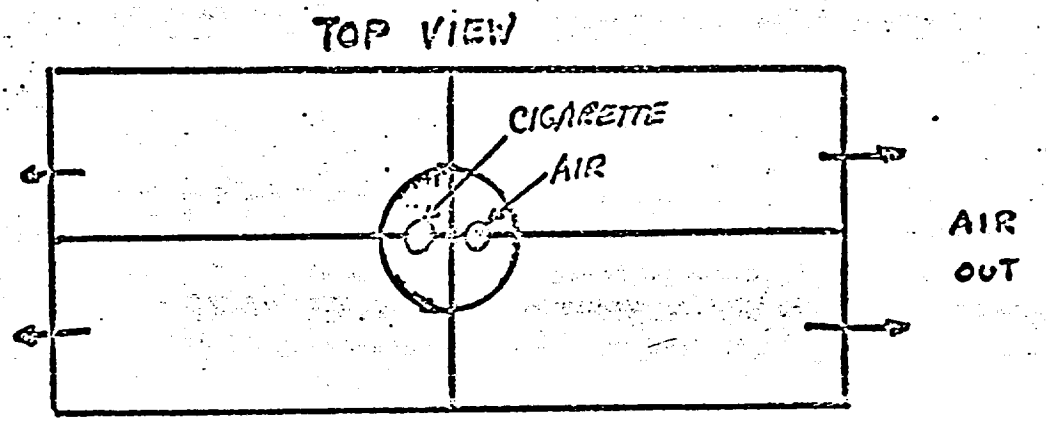
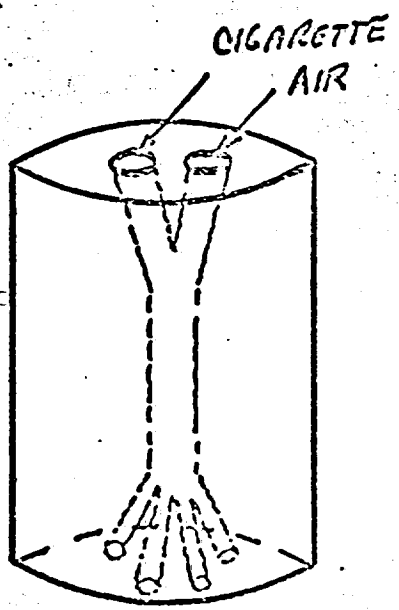
During the isotopic studies animals are housed in special cages as illustrated below; future studies will be made using the modified inlet system shown.





INLET SYSTEM (MODIFIED), SIDE VIEW

Figure 2

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Air is moved through the chamber by vacuum or a small pump and the two valves are electromagnetically operated through a timer, which allows room air or pollutant to enter the chamber in alternating cycles. When smoking the timing is adjusted to obtain a 30 cc puff at -30 cm H_2O pressure (2 secs each minute). In current runs, experimental animals are exposed to tobacco smoke from 20 unfiltered cigarettes per day for 3 days prior to isotope injection. They are continued to exposure at this rate to the time of sacrifice. Subsequent studies will be done with longer periods of exposure to tobacco smoke and other pollutants. In other experiments the intensity of exposure will be varied. The rats will be weighed before the start of exposure and daily thereafter and their food consumption recorded. If the experimental animals show significant differences in food intake from controls (non-pollutant exposed) additional pair fed control animals will be used.

Certain artifacts of the present chamber design which derive primarily from the large surface/volume ratio (which increases drastically when a rat is placed in the chamber) make it difficult to account for all losses and concentration gradients within the chamber. In the current studies this is especially true of condensation nuclei counts, for example, where the counter we are using has a high sampling rate (300 ml/min) and a relatively slow (~1 sec) response time.

A new chamber is under construction using currently recommended techniques (64,79), it will permit a variable S/V-ratio (Figs. 3 & 4). This chamber will be provided with suitable means of feeding and cleaning and concentration measurements for selected substances. It will permit a considerable variation in gas residence time (this is important for the study of periodic variations - diurnal, for example - as well as for the accounting of loss rates to the walls). Although we do not propose to construct a smog chamber some of the recent findings from smog chamber design and measurements are pertinent (80,81). Filtration techniques and efficiencies for smokes and aerosols have been discussed in detail (82,83,84).

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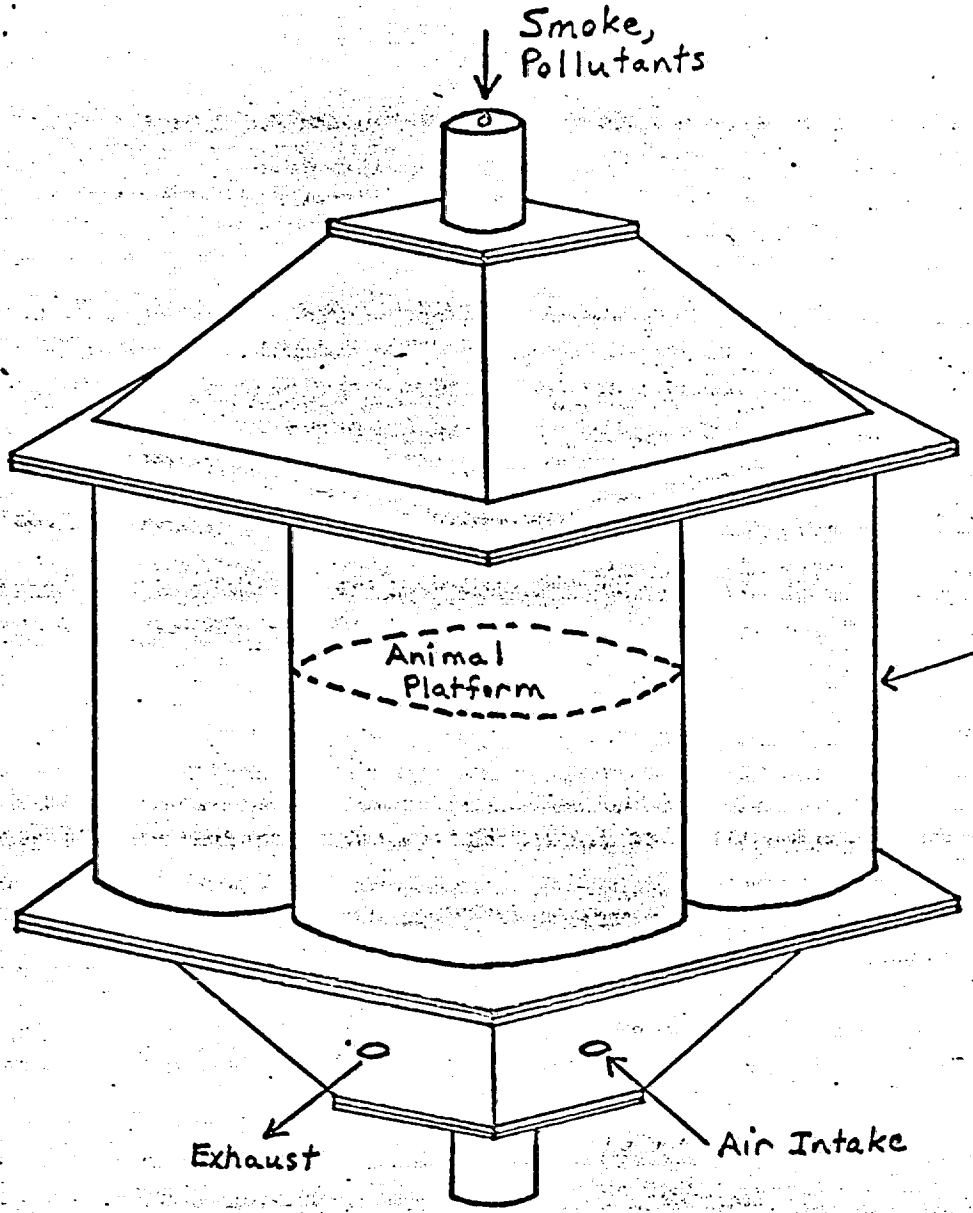

Figure 3

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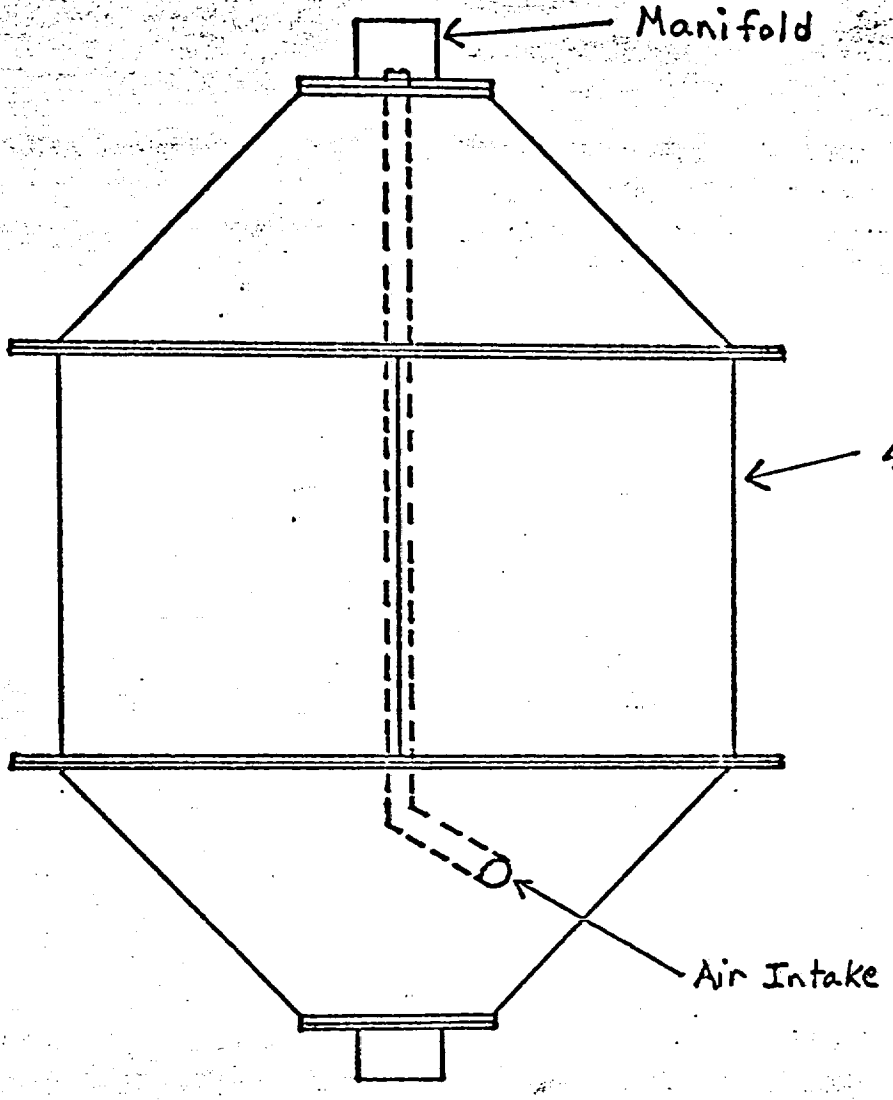

Side View

Top View

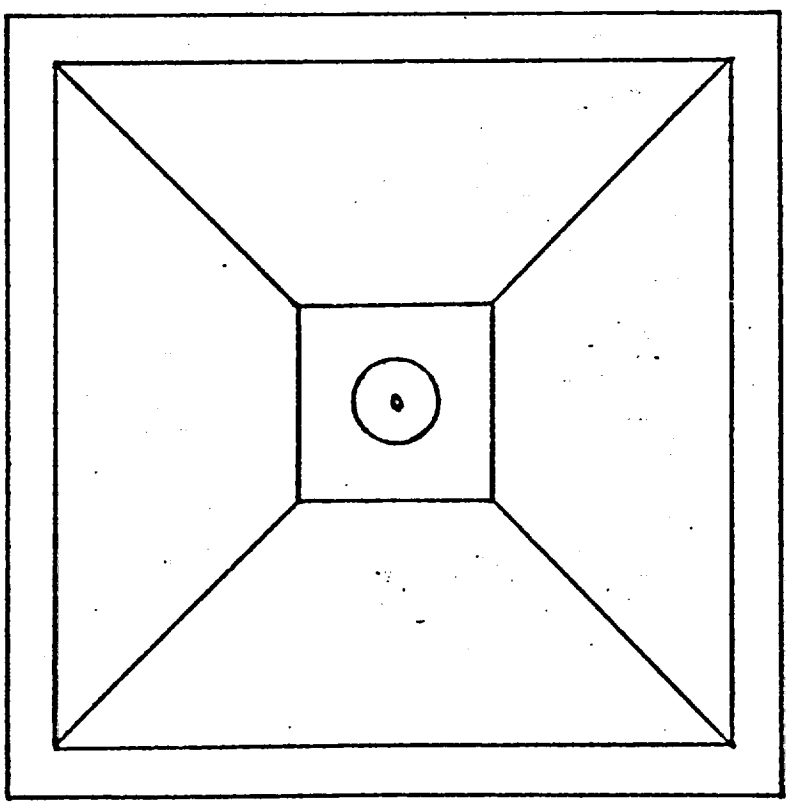

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Figure 4

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1. Experiments on Biosynthesis and Turnover of Surfactant:

General: Adult male rats (Wistar or Long Evans) will be used and after initial treatment as outlined above each will be injected via the tail vein with isotopic mixtures as outlined below and killed by exsanguination by cardiac or aortic puncture at intervals from 1 to 150 hours following isotope injection. At sacrifice an endotracheal tube will be passed and surfactant obtained by lavage using 20 mM Tris (pH 7.5) in 1.15 M NaCl (35). Cells and debris will be removed from this wash by low speed centrifugation and surfactant obtained by high speed centrifugation as described by Pruitt et al (39). Cells will be examined microscopically and chemically in virus experiments. The lung, heart and liver will then be removed and carefully rinsed free of blood. For lipid analyses the tissues will be extracted as described previously (9). Lipids extracted from the various tissues will be analyzed using silicic acid column chromatography and thin layer chromatography to obtain pure triglycerides, 1,2 and 1,3-diglycerides lecithin and phosphatidyl ethanolamine (PE) by methods in standard use in this laboratory (8,9) and/or with two dimensional TLC (85). Purified lecithin and PE will be further sub-fractionated according to the degree of unsaturation of their fatty acids by argentation chromatography (8,86) or by mercuric acetate adduction (40) Completeness of these separations will be checked by gas liquid chromatography of the methyl esters as previously described (2). In studies of the methylation pathway monomethyl and dimethyl PE will be isolated (87,88). Radioactivity of the isolated compounds will be determined by liquid scintillation spectrometry using a toluene, a toluene-TritonX100 or a dioxane based scintillation fluid as appropriate (2).

2. Other Determinations to be made are the Following:

1. Tissue wet weights
2. Pool size of total lipids, total phospholipids, lecithins and phosphatidyl ethanolamines.

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3. Specific activities (dpm/μ mole lipid phosphorus) of each phospholipid and their water soluble phosphorylated precusors.

4. Pool size of water soluble precursors if necessary by reverse isotope dilution as described (4).

5. Electron microscopic evidence of change in lamellar bodies, with autoradiography if indicated, appearance of surfactant lining layer and evidence of increased phagocytosis of lipid material by macrophages.

An aliquot of lung will be extracted as above, another aliquot taken for electron microscopy and the remainder homogenized and submitted to gradient centrifugation to obtain intracellular surfactant type lecithins as described by Pawlowski et al (36) or King and Clements (40). In other groups of rats similarly treated, suitable aliquots of lung, heart and liver will be taken for extraction in 70% ethanol for analysis of the water soluble precursors of lecithin and PE (phosphoryl choline, CDP-choline, phosphoryl ethanolamine and CDP-ethanolamine) as previously described (3,4). For electron microscopy tissue and surfactant will be processed as described by Finley et al (53).

Statistical analysis where applicable will be done using Students "t" test, the Mann-Whitney "U" test and isotope decay slopes will be drawn using the least square method with 95% confidence limits being ascertained for each slope. These programs have been computerized.

3. Specific Experiments: Turnover and Exchange Experiments:

Rats will be housed as described above and fed regular rat chow. The experimental group will be given synergistic mixtures of pollutants to breathe as outlined above. Control rats will be similarly housed and receive air instead of pollutants. After three or more days each rat will receive via tail vein suitable amounts of the isotopic compounds given below and then killed at intervals up to 150 hours. The results from these studies will be used to project longer time periods for future studies.

Based on our present data as reviewed in the Introduction, we believe that 2-^{3}H-glycerol and 1-^{14}C-palmitate (or 9-10-^{3}H-palmitate) are ideal tracers for surfactant turnover studies. These two isotopes will, therefore, be used as

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standard tracer substances. In addition, normal and pollutant exposed animals will also be studied using D_2 labeled palmitic acid. This stable isotope tracer has been chosen for the following reasons:

1) Because it is uniformally labeled, there are 32 deuterium atoms/molecule thus giving a very high signal to noise ratio on mass spectrometry.

2) Palmitic acid from surfactant and other lecithins can be readily separated by methylation and isolation of the methyl esters.

3) The methyl esters are volatile at temperatures used for mass spectrometry.

4) Methyl esters are readily quantitated by gas chromatography.

In addition to labeled glycerol and palmitate, experiments will be performed using ^{14}C-leucine to examine the relative relationship of lecithin and protein turnover in the surfactant system. The system will be isolated by either the method of Pruitt et al (39) or King and Clements (40). In view of the recent studies of Naimark (41) we will pay particular attention to the pulmonary alveolar macrophages. These cells will be isolated from lung lavage fluid (41) and their lipids extracted and analyzed as described above. Since such cells exhibit active phospholipid metabolism of their own, quantitative data on their catabolism of "ingested" surfactant may be difficult. The following approaches to this problem will be tried:

1) Comparison of total numbers of macrophages recovered in lung lavage fluid in normal and pollutant exposed rats at each time period (i.e. cells/mm^3 of lavage fluid). A constant volume (3 x 6 ml) of fluid will be used for lavage.

2) Comparison of number of macrophages per alveolus as judged by light microscopy in normal and experimental animals. This method will be a check on #1.

3) DPL will be isolated from the macrophages in the lavage fluid and total dpm in DPL/lavage volume, as well as specific activity (dpm/μmole DPL) determined.

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These observations will be correlated with changes in metabolic rate of DPL in pollutant exposed animals in order to try to establish whether the changes in metabolic rates are secondary to stimulation of macrophage activity and/or macrophage population in the alveolae.

Studies will be performed to establish a standardized lavage procedure for recovery of surfactant lecithin. The procedure currently being used (39) yields fairly consistent recoveries of about 1 μmole lecithin/rat, using 3 washes of 6 ml each. In order to be able to measure secretory rates more precisely it will be necessary to refine this method further. We will, therefore, compare the more recently published method of Young and Tierney (96) with our present system. By these means it should be possible to examine secretion rates of surfactant DPL in relation to pollutant exposures.

Since controversy still exists regarding the relative importance of the Kennedy and the methylation pathways to lecithin biosynthesis in the lung, it will be important to examine these two pathways after pollutant exposure, if exposure to pollutants results in more rapid turnover of surfactant DPL. Studies will be performed using methionine-methyl-^{3}H, or ethanolamine-1-2-^{14}C to evaluate the methylation pathway contribution, if any, to DPL biosynthesis in normal and experimental rats. Since this pathway is of established importance in liver, but of doubtful significance in lung, these studies will be performed in the isolated heart lung preparation to avoid any problems with uptake by lung of lecithin secreted by the liver into the circulation.

Based on the present rates of smoking of the rats it is not possible to predict what effect increased amounts of cigarette smoke or variable concentrations of other pollutants, etc. will have on the phospholipids in these tissues, especially since no relationship between pollutant concentrations and the amounts inhaled has been established. For this reason experiments in the larger chamber to be built will be more meaningful since a correlation between pollutant concentration in the chamber and amount inhaled should be more readily established. Experiments using various exposure times (current) must be coupled with varying concentrations.

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Some work with single pollutants will be carried out under the current NIH-grant. Of more interest will be studies with pollutants (such as SO_2) which are largely removed in the upper airways (89,90); if these produce a response (increased turnover rates after correction for increased respiratory rate, if any) an indirect effect must be operative; this could be sorted out by synergistic studies such as sulfur dioxide in the presence of oxidizing (transition metal salts) nuclei. Toxicity of long-term exposure to sulfuric acid mist has recently been reported (91).

Techniques for the preparation of controlled test atmospheres are routinely in use in one of our laboratories (ERA). These are done via dynamic syringe, rotating stopcock, permeation tube, and the use of plastic bags. Where possible continuous monitoring of pollutants will be performed (O_3, NO, NO_2 -chemi-luminescense; particles via CN-counter or mass sizing; SO_2-via hydrogen peroxide or photometric).

Experiments with Essential Fatty Acid (EFA) deficient rats are currently underway. The combination of EFA-efficiency and pollutant exposure upon the synthesis, storage, and secretion of the surfactant system will be extended to other pollutant systems if present studies warrant it.

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4. <u>In Vitro Experiments</u>

As phospholipid synthesis in cell free systems is not always the same as in intact animals, further studies will be performed using isolated heart-lung preparations. Such a system has been described in the rat by Simpson-Morgan (78). Heart-lung preparations prepared by this method will be obtained from normal and pollutant exposed animals and incorporation of suitable radioactive precursors after intracardiac injection followed for 1-4 hours in lung and heart by methods described above. These steps will permit study of surfactant synthesis in a system where hepatic synthesis of phospholipid and protein are excluded, thus allowing us

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to rule out any hepatic contribution. These studies will also avoid the problems associated with adipose tissue lipolysis secondary to nicotine stimulation.

Electron Microscopy will be used to evaluate the following:

1. To help ascertain purity of cell fractions.

2. To compare the appearance of macrophages in normal, EFA deficient and pollutant exposed animals looking particularly for evidence of phagocytosis of membrane structures resembling surfactant.

3. To examine the Type II alvelor cells in normal, EFA deficient and pollutant exposed animals with particular attention to the appearance both qualitatively and quantitatively of the lamellar bodies. It is hoped that such observation, coupled with biochemical data, will allow better understanding of the assembly and secretion of the surfactant system, particularly in relation to the membrane systems concerned with secretory granules and protein synthesis in EFA deficient animals.

D. SIGNIFICANCE

1. Do pollutants increase the turnover of surfactant lecithin? If this is the case the slope of disappearance from the surfactant of pollutant-exposed rats should be significantly greater than that of control animals. This is what we are finding currently with cigarette smoke. Long-term exposures of rats to high levels (2.9 ppm) of NO_2 resulted in a 13% decrease in lung compliance, a significant reduction in surface-active properties (increased surface tension) an 8.7% decrease in lung lipid content, and a marked decrease in percentage of total saturated phospholipid fatty acids (51). It was suggested that lung instability (P-V measurements) with NO_2-exposure was due to alterations in fatty acid composition of surfactant phospholipids. It is clearly desirable and necessary to combine turnover measurements with phospholipid fatty acid analysis from lavaged lungs if underlying mechanisms are to be understood. The experiments proposed should also shed light on the effect of pollutants on the phospholipid metabolism in heart and liver.

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2. Long term exposure studies are thought to be more significant than "forced" inhalation studies. Precise knowledge of the effects of various environmental factors upon the metabolism and function of the surfactant system is lacking. Epidemiological studies indicate that such factors play an important role in the pathogensis of acute and chronic pulmonary disease in man.

   Other factors have to be accounted for. Kerr (92) has shown that there is in man a diurnal variation of respiratory function independent of air quality; i.e. a chemical or metabolic change (for any observed changes) would be indicated in our experiments, rather than a mechanical one. However, we have not verified such a diurnal variation in the rat.

3. Which of the three pathways of lecithin synthesis is involved in surfactant production? In vivo and in vitro studies from control and pollutant exposed animals will determine which pathway shows increased activity in conformity with increased in vivo turnover. How do the findings relate to the surfactant pool?

4. Studies in man

   Once the techniques for use of stable isotopic tracers have been developed based on the work done by Klein and co-workers (93,94) in the animal models described above we will apply their techniques to studies in healthy human subjects and patients with bronchopulmonary disease. Techniques for obtaining pulmonary lavage fluid for man have been developed by others and can be applied to these studies. Precise description of experiments in man will have to await more precise definition of the problem in animal studies. We anticipate that such human studies will not therefore be undertaken for at least two years. At that time a precise protocol will be submitted for review both by the granting agency and the Committees or Experimentation Involving Human Subjects of the Albany Medical College.

5. Clements (95) presented an interesting correlation of surface active material vs. pulmonary surface area for several species (experimentally and theoretically). Based on the theoretical curve all species have a surfactant reserve. This correlation (if the fashion in which it was arrived is accepted) will be important to the interpretation of turnover rates and pool sizes to be obtained from the proposed study.

E. FACILITIES AVAILABLE

The experimental laboratories available for this study include 1000 square feet of area for general medical research and 500 square feet of an air pollution laboratory. There are 350 feet of office area available.

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The following major items of equipment available for this study include: liquid scintillation system (Beckman L S 250), preparatory ultracentrifuge (Beckman L 265), high speed refrigerated centrifuge (Servall RC 2-B), gas chromatograph (F & M Model 400), electron microscope (RCA-EMU), 2 fraction collectors, Packard strip counter, refrigerated microtome (Porter-Blum), gas chromatograph (Perkin-Elmer 900), gas chromatograph (Gow-Mac), ozone generator (Welsbach), ozone meter (Mast), spectrophotometers (B & L Spectronic 20, 70, 88), ultraviolet spectrophotometer (Beckman DU 2), NO_x analyzer (Scott 225 Chemiluminescence) atomic absorption spectrometer (Beckman), total carbon analyzer (MSA), integrating nephelometer (MR), condensation nuclei counter (Environment/One Rich 100), Lundgren impactor (Environmental Sciences), and radioactive Isotope measuring equipment. The following items are available -- to be shared with other investigators: Digital Equipment Corporation PDP-12 computer, NCR Century 200 computer, IBM 360/50 computer, nuclear magnetic resonance and electron spin resonance instrumentation, and mass spectrometry (Dr. Hudson's laboratory at RPI) and neutron activation analysis equipment.

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